RECOMBINANTS OF AVIAN RNA TUMOR VIRUSES: CHARACTERISTICS OF THE VIRION RNA

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Summary

The RNAs of several avian tumor virus recombinants which had inherited their focus forming ability from a sarcoma virus and the host range marker from a leukosis virus were investigated. Electrophoresis and analysis of oligonucleotide fingerprints showed that the cloned sarcoma virus recombinants contained only size class a RNA, although they had acquired a marker which resided on class b RNA in the leukosis virus parent. Class a RNA of different recombinant clones, derived from the same pair of parental viruses and selected for the same biological markers, differed slightly in electrophoretic mobility from each other and from the parental sarcoma virus. Small electrophoretic differences were also observed between the class a RNAs of various strains of avian sarcoma viruses and between class b RNAs of leukosis viruses, but these minor variations in RNA size were not related to the size of recombinant RNAs derived from these viruses.

Recombinants of the same cross and selected for the same pair of markers were also found to have different fingerprints of RNase T1 resistant oligonucleotides. The average complexity of the 60-70S RNA prepared from wild type sarcoma viruses was estimated to correspond to 2.7×10^6 daltons, suggesting that the genome of RNA tumor viruses is polyploid.

All these observations led us to propose that recombination among avian tumor viruses occurs by crossing over between homologous pieces of nucleic acid.

Introduction

Nondefective avian sarcoma viruses can undergo high frequency genetic recombination with avian leukosis viruses (1,2,3). Since the 60-70S RNA of avian tumor viruses consists of several pieces (4), it appeared likely that this recombination represented reassortment of markers situated on different genome subunits. However preliminary electrophoretic analysis of the RNA from a recombinant between PR-B sarcoma and RAV-3 leukosis virus led us to propose that recombinants between avian tumor viruses originate from crossing-over (5,6).

This proposal was based on the following argument: Cloned nondefective sarcoma viruses contain only 30-40S RNA of size class *a*. The 30-40S pieces of leukosis viruses are of the smaller size class *b*. In a cross between PR-B sarcoma and RAV-3 leukosis virus, the recombinant is selected for the focus forming ability of the sarcoma virus linked to the host range marker of the leukosis virus, thus combining markers which on parental RNAs are situated on size *a* and *b* molecules respectively. However, the RNA of the PR-B x RAV-3 recombinant contained only class *a* pieces, and thus the leukosis-derived marker must have become incorporated into class *a* RNA.

To distinguish better between reassortment and crossing over, additional recombinants which had inherited the focus forming marker from a sarcoma and the host range marker from a leukosis virus were investigated. The RNAs of recombinants and of parental viruses were compared with respect to electrophoretic mobility, RNase T1 fingerprint pattern, and genetic complexity. The results favor crossing over and suggest that the genome of RNA tumor viruses may be polyploid.

Results

The RNAs of several recombinant sarcoma viruses obtained from different host range crosses.

Figure 1A shows an electropherogram of heat dissociated 60-70S RNA from a recombinant between the focus forming marker of PR-A and the host range marker of RAV-2. This recombinant contained only 30-40S RNA of class *a*, which coincided with the class *a* RNA of the PR-C standard (the latter showed a class *b* component as well, probably representing a transformation-defective segregant which had formed during nonclonal passage of this virus stock) (5,7,8). The RNA of a recombinant between PR-B focus for-

mation and RAV-1 host range was co-electrophoresed with the PR-A x RAV-2 recombinant of Figure 1A, and the electropherogram is presented in Figure 1B. Both viruses contained only 30-40S RNA of size class *a*. Five other recombinants between the focus forming marker of a sarcoma and the host range marker of a leukosis virus were studied and found to contain only class *a* RNA. Some of these recombinants showed the presence of class *a* and class *b* RNA initially; however, subsequent cloning eliminated class *b* RNA. We conclude that probably all recombinants carrying the focus forming marker of a sarcoma and the host range marker of a leukosis virus contain only 30-40S RNA of class *a*.

Further evidence for the absence of class b RNA from the 60-70S complex of sarcoma virus recombinants.

Heated 60-70S RNA of the recombinants investigated contains, in addition to a major component of 30-40S RNA, minor heterogeneous RNA species of variable concentrations (Figures 1-4). Therefore, it may be argued that these minor RNAs are distinct subgenomic fragments, including class *b* RNA of the parental leukosis virus perhaps acquired by reassortment. To test this possibility, heat dissociated 60-70S ^{32}P RNA of PR-A x RAV-2 (Fig. 1A) was fractionated by sedimentation. Fractions comprising the 30-40S RNA species and fractions comprising the minor heterogenous species sedimenting at <30S and >10S were pooled separately (Fig. 2A) and studied chemically. A sensitive method for partial sequence comparison of RNAs has been developed by Brownlee and Sanger (9) and is based on the electrophoretic and chromatographic properties of RNase T1 resistant oligonucleotides. The technique has been used recently to compare tumor virus RNAs (8). It is shown in Figs. 2B and 2C that the fingerprints of RNA pool 1 (>30S) and of RNA pool 2 (<30S and >10S) were indistinguishable. We conclude that the minor heterogenous RNA species obtained after heat-dissociation of 60-70S RNA consist predominantly of degraded 30-40S RNA of size class *a* rather than of chemically distinct RNA species. However, the presence of low (<10%) concentrations of small RNA species unrelated to 30-40S RNA cannot be excluded by this experiment.

Different recombinants derived from the same pair of parental viruses and selected for the same markers have RNAs of different size.

Only two (host range and focus forming markers) of presumably several genes, which may be exchanged between leukosis and sarcoma viruses, have been selected for in the recombinants studied here. If crossing over takes place

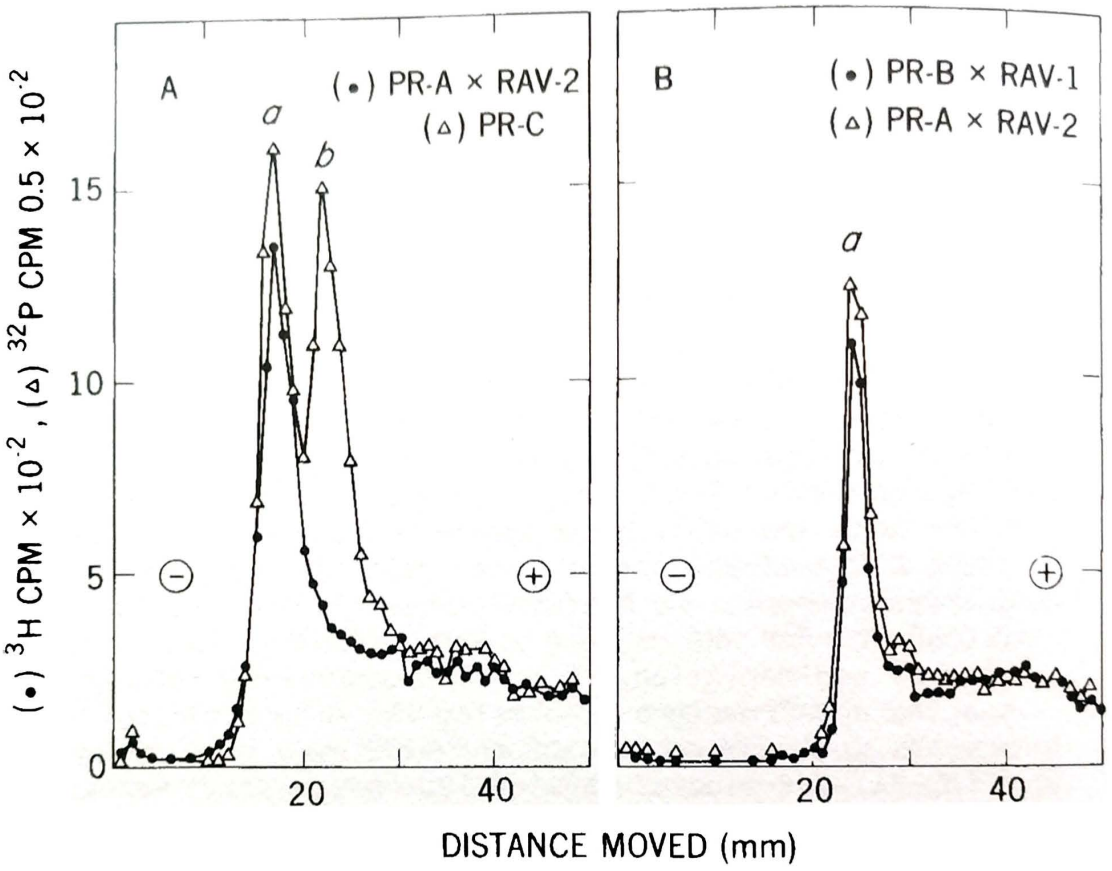

Fig. 1. Electrophoresis of heat-dissociated 60-70S RNA of two cloned recombinant sarcoma viruses, PR-A x RAV-2 and PR-B x RAV-1. (A) Appropriate amounts of radio-labeled PR-A x RAV-2 RNA and PR-C RNA were mixed and heated in electrophoresis sample buffer and subjected to electrophoresis in 2% polyacrylamide as described (5). PR-C had not been cloned recently and contained both class *a* and class *b* RNA species. (B) A mixture of the RNAs of two sarcoma virus recombinants PR-B x RAV-1 and PR-A x RAV-2 was analyzed as described for A.

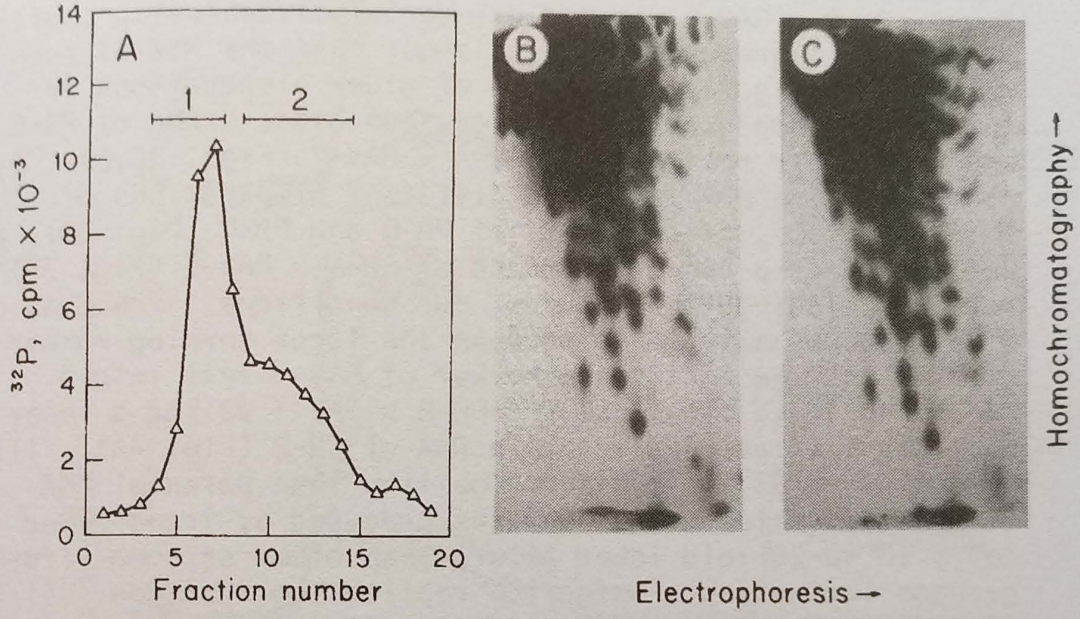

Fig. 2. Sedimentation and fingerprint analyses of heat-dissociated 60-70S ^{32}P-RNA (approx. 2 x 10^6 cpm) of a recombinant sarcoma virus PR-A x RAV-2, harvested at 3-5 hour intervals from infected cells. (A) RNA in 300 µl was heat-dissociated as described for Fig. 1. After addition of NaCl to 0.1 *M* the solution was layered on a 15-30% glycerol gradient containing 0.1 NaCl, 0.01 Tris HCl pH 7.4, 1 m EDTA and 0.1% sodiumdodecylsulfate. Centrifugation was for 105 minutes at 50,000 rpm in a Spinco SW 50.1 rotor at 20°C. Fractions indicated by the bars in Fig. 2A were combined in two pools and the RNA was ethanol-precipitated. Fingerprinting of RNA pools 1 (B) and 2 (C) was as described previously (8).

between RNA tumor virus genomes, it may theoretically occur at any point on the genetic map between the focus forming and the host range markers. In this case, the RNAs of recombinants selected for the same markers, but derived from different cross over events could differ in their sequences. The first indication of such a difference was the observation that the 30-40S RNA of a recombinant between PR-B and RAV-3 (PR-B x RAV-3 #1) had a lower electrophoretic mobility and was therefore probably larger than the RNA of parental PR-B (Fig. 3A). The RNA of other preparations of this recombinant was also larger than class *a* RNA of PR-C and of another recombinant, PR-A x RAV-2 (Figs. 3C,E). By contrast class *a* RNAs of two different preparations of PR-B (Fig. 3B), class *a* RNAs of PR-B and PR-C (Fig. 3D), as well as class *a* RNA of PR-B and of PR-A x RAV-2 (Fig. 3F) were not distinguishable under our conditions. The RNAs of other recombinant clones between the focus forming marker of PR-B and the host range marker of RAV-3 fell into 3 electrophoretic classes: (i) PR-B x RAV-3 #2 had a lower mobility than parental class *a* RNA of PR-B (Fig. 4A). (ii) PR-B x RAV-3 #3 had a higher mobility than parental RNA (Fig. 4B). This recombinant was produced by transformed cells in 10-20 fold lower titers than other sarcoma viruses, perhaps indicating a defective replicating function. (iii) PR-B x RAV-3 #4 had practically the same mobility as parental PR-B RNA (Fig. 4C). These experiments indicate that the primary structure of recombinant RNAs differs from that of parental RNA.

The apparent molecular weight by which certain recombinant RNAs differ from parental, wild type RNA is estimated to be around 70,000 daltons on the following basis: The electrophoretic differences observed between RNAs were ± one fraction (Figs. 3,4). Class *a* and class *b* RNA differ by about 5 fractions under the same condition (*cf* Figs. 1, 3) (5,10). The difference between *a* and *b* was estimated to be about 350,000 daltons (10). Thus certain recombinant RNAs differ from wild type RNA by about one-fifth of that or 70,000 daltons. The size differences observed among the RNAs of distinct recombinants were stable after several successive clonings. This suggests that the size variations are not likely to be host modifications similar to those observed earlier in two specific cases which were not stable on passage of the virus in different cells (5).

The exact size of recombinant RNA cannot be predicted from the size of parental RNAs.

Class *a* RNAs of different nondefective sarcoma viral

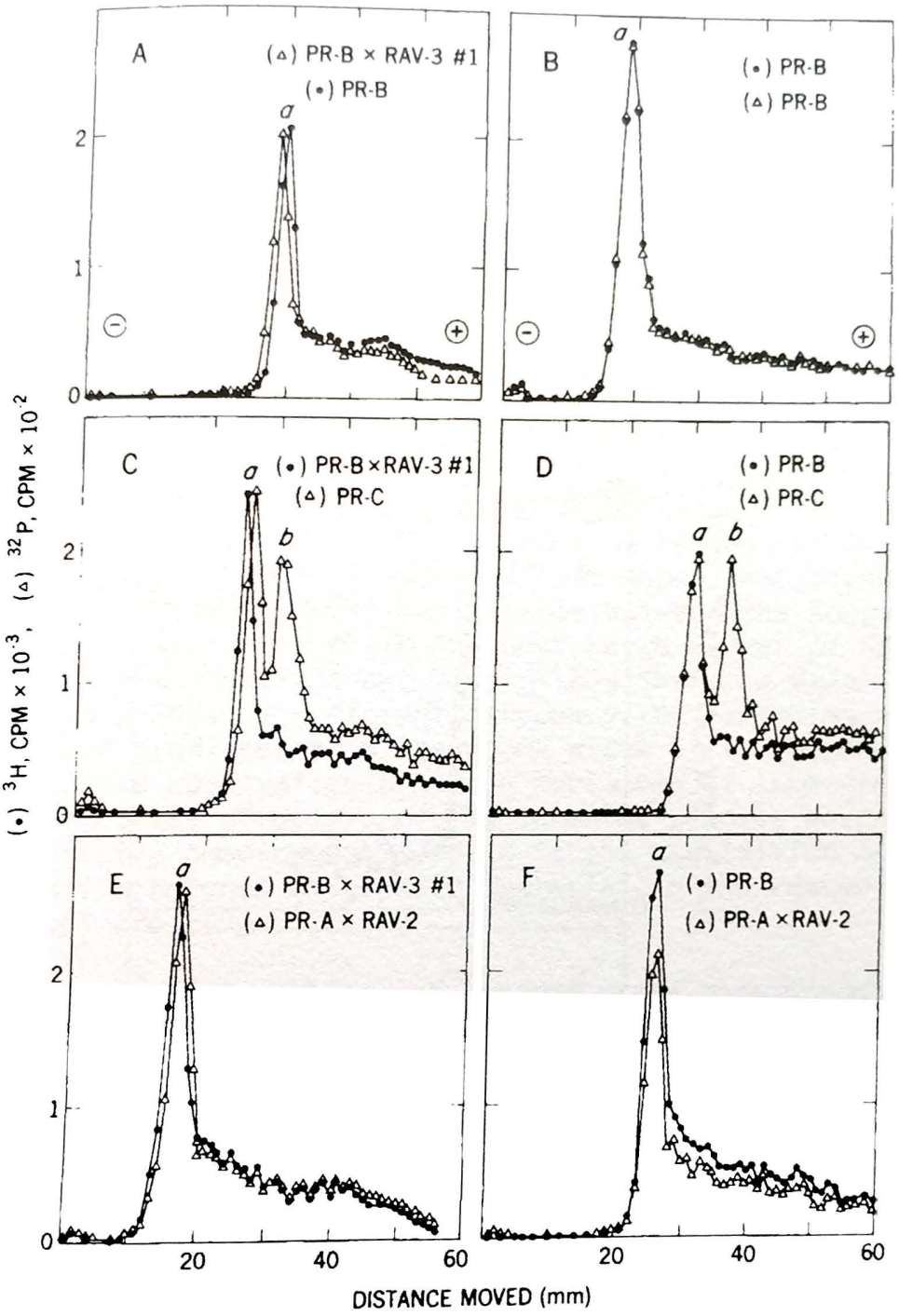

Fig. 3. Heat-dissociated 60-70S RNAs of different preparations of a cloned recombinant sarcoma virus, PR-B x RAV-3 #1 and of several other wild type and recombinant sarcoma viruses after electrophoresis as described for Fig. 1. The experiments were carried out to demonstrate that class *a* RNA of PR-B x RAV-3 #1 had a lower electrophoretic mobility than other class *a* RNAs as described in t the text.

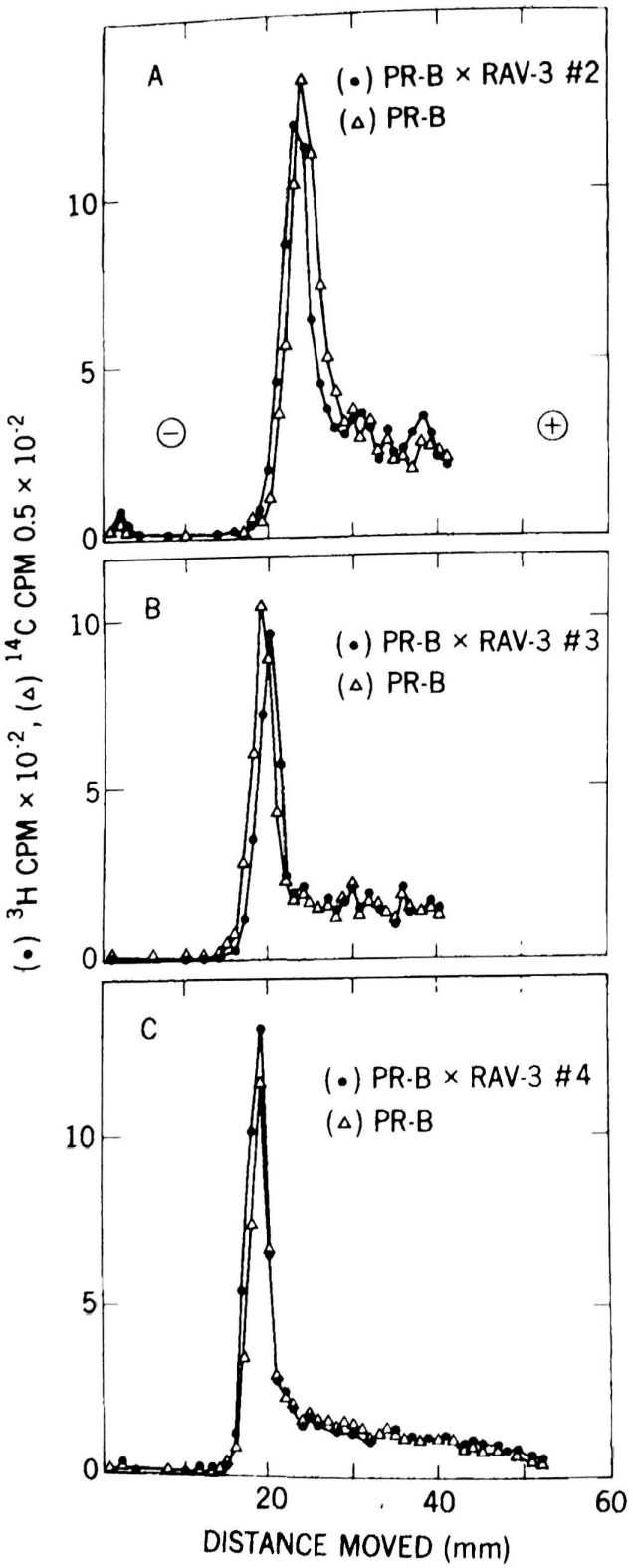

Fig. 4. The RNAs of three different recombinants PR-B x RAV-3 #2 (A), #3 (B) and #4 (C) after heat dissociation and electrophoresis with a standard of PR-B RNA. Conditions were as described for Fig. 1.

strains were shown to differ electrophoretically by about one fraction (5); likewise class *b* RNAs of different leukosis viruses differ slightly if compared by this method (Fig. 5). Since the class *a* RNAs of PR-A, PR-B and PR-C are all electrophoretically indistinguishable (Fig. 3, ref. 5) but the class *b* RNAs of RAV-1, RAV-2 and RAV-3 used to form recombinants with these sarcoma viruses are different, it appeared possible that a direct correlation existed between the size of class *b* RNA in the parental leukosis virus and the size of class *a* RNA in the recombinant virus. For instance, it was found that compared to a class *b* RNA standard (*td*PR-C), the RNAs of RAV-1 and of RAV-2 are slightly larger, while the RNA of RAV-3 is the same size as the standard. Yet, the RNAs of two recombinants between PR-B and RAV-3 (#1, Fig. 3 and #2, Fig. 4 respectively) were actually larger than a recombinant between PR-A and RAV-2 (*cf.* Figs. 1,3) and a recombinant between PR-B and RAV-1 (*cf.* Fig. 1). Moreover, it was shown that class *a* RNAs of different recombinant clones between the focus forming marker of PR-B and the host range marker of RAV-3 have different mobilities. It follows that the exact size of class *a* RNAs of different sarcoma virus recombinants cannot be predicted from the known sizes of the parental RNAs. This observation could be explained if some cross-overs occurred at points of the parental genomes which were not strictly homologous, leading to the acquisition or loss of small stretches of genetic material in the recombinants (unequal crossing over).

Fingerprint-analyses of sarcoma virus recombinants derived from the same pair of parental viruses and selected for the same markers.

If crossing over is responsible for the small electrophoretic differences observed among the class *a* RNAs of four recombinants between PR-B and RAV-3, it would be expected that these RNAs also differ in their sequences. This possibility was tested by fingerprinting the RNAs of these four recombinants of the PR-B x RAV-3 cross (*cf.* Figs. 3,4). It is apparent that their oligonucleotide patterns are very similar but differ from each other in at least 2-3 out of about 20 major RNase T1-resistant oligonucleotide spots (Fig. 6A-D). Some spots which are found in one but not in all other recombinants are indicated by arrows. The pattern of wild type PR-B is shown in Fig. 6E and that of RAV-3 in Fig. 6F. Their patterns differ from those of the recombinants more extensively than the recombinant patterns differ from each other.

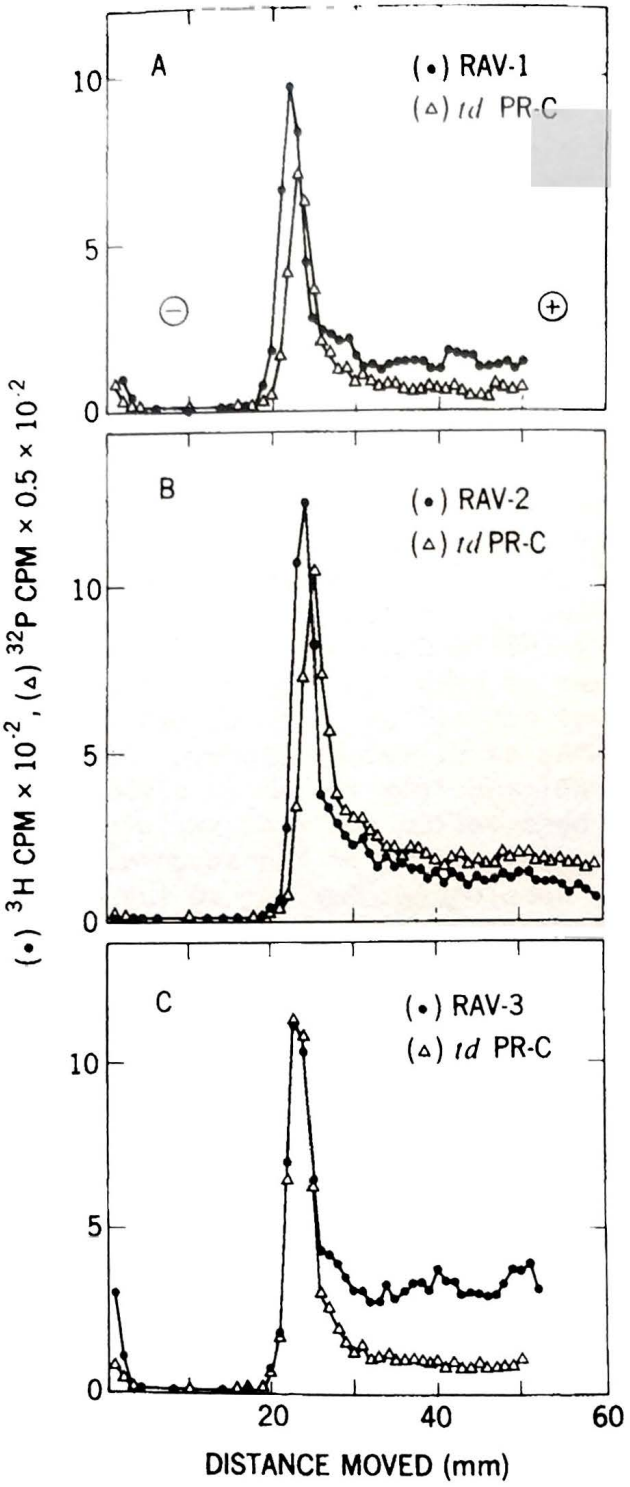

Fig. 5. The RNAs of three leukosis viruses RAV-1 (A), RAV-2 (B) and RAV-3 (C), used to form recombinants with PR RSV strains, after heat-dissociation and electrophoresis with a standard of *td* PR-C RNA. Conditions were as described for Fig. 1.

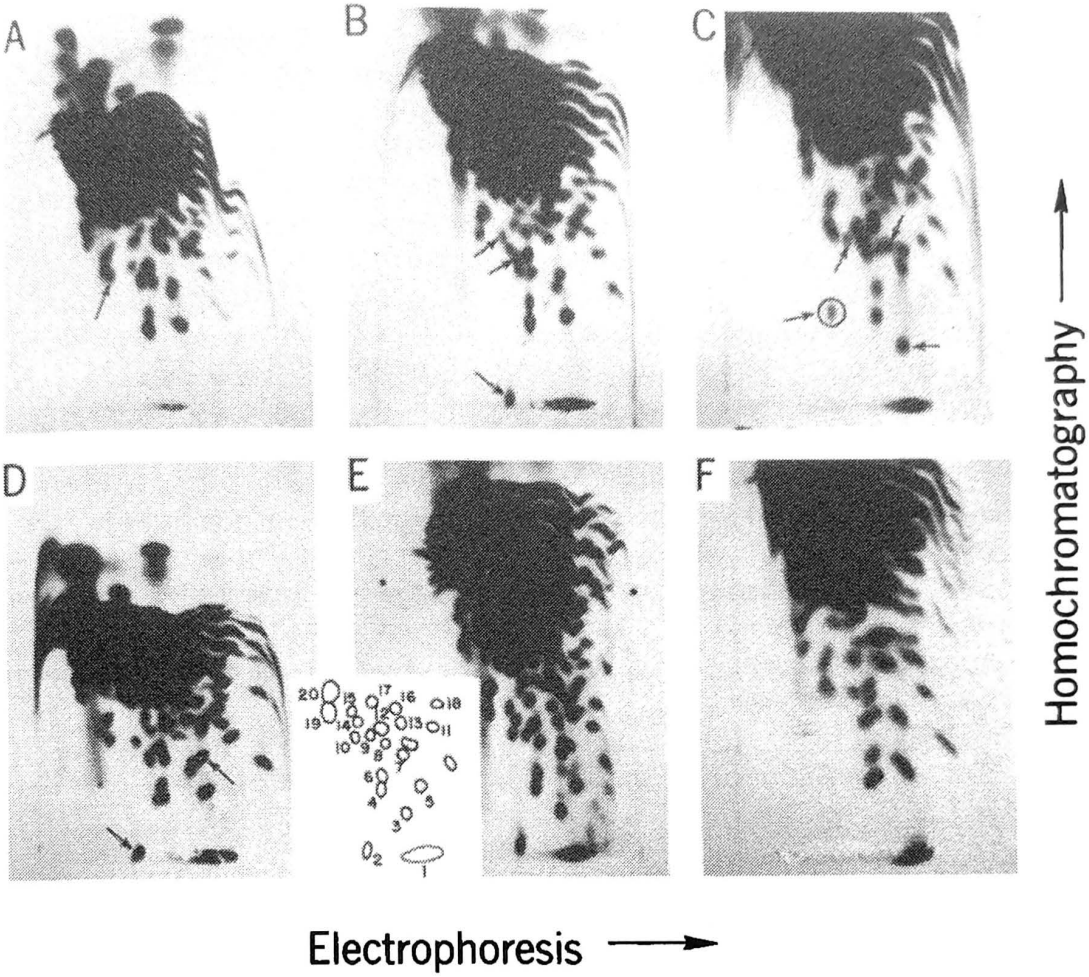

Fig. 6. Fingerprint analyses of the RNase T1-digested 60-70S ^{32}P-RNAs of the four recombinants PR-B x RAV-3 #1 (A), #2 (B), #3 (C) and #4 (D) as well as of PR-B (E) and RAV-3 (F). 60-70S ^{32}P-RNAs of virus harvested at 12 hour intervals were digested and analyzed as described previously (8) except that a 3% homo-mixture b (9) was used. The arrows in A-D indicate spots not found in all of the four recombinants analyzed. The circled spot in C has no homologous counterpart in the patterns of either parental virus (E,F). A schematic tracing of the large oligonucleotides of PR-B (E) identifies spots which were analyzed as described in Table 1.

Although differences observed by fingerprinting are only qualitative (8), it may be concluded that the recombinants analyzed differ in RNA sequences. This observation supports the possibility that crossing over points between focus forming and host range markers are not at a fixed location. We have not determined in detail which of the large oligonucleotides of the four PR-B x RAV-3 recombinants are derived unchanged from either parental viral strain and which of these oligonucleotides contain new sequences representing sites at which crossing over may have taken place. However, at least one spot, circled in recombinant #3, Fig. 6C, appeared to be new and not to have a homologous counterpart in either parental virus (Figs. 6E,F). This spot as well as some others on Fig. 6A-D had lower intensities than neighboring spots of presumably similar size (9). This may be due to incomplete transfer of the oligonucleotides from the cellulose acetate strip used for electrophoresis to the DEAE thin layer used for homochromatography (9). It may also reflect lack of homogeneities in the RNAs. Further work, including complete transfer of oligonucleotides, as used in Figs.6E,F will be required to resolve this problem.

The 60-70S tumor virus RNA appears to be largely polyploid.

If the 30-40S subunits of a given 60-70S tumor virus RNA were identical and the 70S RNA represented a polyploid genome, stable recombinants could arise only by crossing over. The genetic complexity of the 60-70S RNA should then be equal to that of each of the 30-40S pieces. However, if 60-70S RNA were haploid, its complexity would be higher than that of an individual 30-40S subunit. The complexity of an RNA species uniformly labeled with ^{32}P can be estimated if the sizes of several oligonucleotides derived from it are determined, and the radioactivity of these oligonucleotides is compared with the total radioactivity in the intact RNA molecule (11). The average complexity of PR-B RNA as determined from about 20 RNase T1-resistant oligonucleotides, resolved as described in Fig. 6E, amounted to 2.7×10^6 daltons (Table 1). This is in good agreement with the lower of several molecular weight estimates for viral 30-40S subunits obtained by other methods (10). Preliminary experiments suggest that the RNA of a recombinant has a similar complexity. Further work will be required to explain the fluctuations ($\sigma = \pm 0.48 \times 10^6$ daltons) observed among complexity-estimates based on different oligonucleotides. These may be due to inhomogeneities of the

TABLE 1

THE COMPLEXITY OF PR-B RNA* ESTIMATED FROM THE SIZES OF RNASE T_1-RESISTANT OLIGONUCLEOTIDES

Oligonucleotide spot no.†	CPM		Approximate base composition		Calculated complexity of RNA in daltons ($\times 10^{-6}$)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
1	40,370	39,600	Poly A	Poly A	-	-
2	8,200	-	$(C_3A_7UG)_2$§	$(C_3A_6UG)_2$	3.2	2.9‡
3	6,500	-	$C_3A_3U_4G$	$C_4A_4U_4G$	1.8	2.1
4	6,060	4,900	$C_6A_4U_3G$	$C_5A_3U_2G$	2.5	2.1
5	7,900	-	$(C_3A_2U_3G)_2$	$(C_3A_2U_4G)_2$	2.4	2.7
6	5,890	4,800	$C_5A_3U_3G$	$C_4A_3U_2G$	2.2	1.9
7	7,050	-	$(C_3A_3U_3G)_2$	$(C_3A_3U_3G)_2$	3.0	3.0
8	5,470	-	$C_4A_4U_3G$	$C_4A_5U_3G$	2.4	2.6
9	4,560	-	$C_4A_3U_3G$	$C_5A_4U_3G$	2.6	3.1
10	4,160	-	$C_5A_3U_2G$	$C_6A_4U_2G$	2.8	3.3
11	4,660	-	C_3AU_5G	C_3AU_5G	2.3	2.3
12	9,000	-	$(C_5A_3U_4G)_2$	$(C_5A_4U_3G)_2$	3.1	3.1
13	5,250	4,500	$C_2A_3U_3G$	$C_3A_4U_3G$	1.8	2.3
14	4,490	-	$C_4A_3U_2G$	$C_5A_4U_2G$	2.4	2.9
15	3,880	-	C_3A_3UG	$C_4A_4U_2G$	2.2	3.0
16	4,150	4,300	C_4AU_3G	-	2.3	-
17	5,940	5,000	$C_3A_4U_3G$	-	1.8	-
18	4,580	-	$C_2A_2U_4G$	$C_3A_2U_6G$	2.1	2.8
19	10,800	8,800	$(C_5A_4UG)_3$	$(C_4A_4UG)_3$	3.3	3.1
20	11,900	9,200	$(C_4A_4UG)_3$	$(C_4A_5UG)_3$	2.7	3.3
				Average¶	2.6	2.8

*60-70S ^{32}P-RNA derived from virus harvested at 12-hour intervals was prepared and exhaustively digested with RNase T_1 as described (8). The digest was resolved by electrophoresis and, after complete transfer to DEAE-cellulose, chromatographed as shown in Fig. 6. Two identical patterns were made each using 3.34×10^6 cpm (Exp. 1) or 2.86×10^6 cpm (Exp. 2) of the digested RNA. One pattern was used to determine the total radioactivity in a spot and the other to determine base compositions. An average of 380 cpm/nucleotide was found in Exp. 1. Oligonucleotides were eluted and base compositions determined by published procedures (9). Further details will be described elsewhere (Beemon and Duesberg, in preparation). The complexity was calculated using an average nucleotide MW of 323, calculated from the base composition of PR-B RNA (24.4% C, 23.8% A, 28.8% G, 23.0% U) and the known MW of the nucleotides.

†Numbers refer to diagram in Fig. 6E.

‡CPM from Exp. 1 and base compositions obtained in Exp. 2 were used in all calculations except where CPM from Exp. 2 are shown.

§Specific activity indicates more than one G per oligonucleotide due to either 2 (or 3) unresolved spots or to incompletely digested RNA. Heterogeneity of some spots is also suggested by their autoradiographic appearance; see for example spots #12, 19 and 20.

¶Values of presumed multiple spots are considered multiply in the average.

60-70S ^{32}P RNA prepared from virus harvested at 12 hr intervals (12,13,14). Oligonucleotides deriving from preferentially degraded sequences of 60-70S RNA would lead to a higher complexity-estimate and oligonucleotides derived from sequences of partially degraded RNA which associate preferentially with 60-70S RNA would lead to a lower complexity-estimate. Although these possible sources of error require further investigation we tentatively conclude that the RNase T1-resistant sequences of the 60-70S tumor virus RNA have an approximate complexity of 3×10^6. Thus, the 60-70S viral RNA appears largely polyploid and consequently recombination is likely to involve crossing over.

Discussion

Recombination involves crossing over.

Sarcoma virus recombinants, derived from a sarcoma virus parent with only class *a* RNA and a leukosis virus parent with only class *b* RNA, were found to contain only, or almost only, class *a* RNA. The class *a* RNA of certain recombinants was either larger or smaller than the parental class *a* RNA. Further, oligonucleotide patterns of recombinants, presumably derived from different recombination events but selected for the same markers, differed among themselves, and differed even more extensively from those of parental RNA. The 60-70S RNAs of wild type and of recombinant viruses appeared to be largely or completely polyploid. The sum of all these observations favors the conclusion that at least some of the recombination among avian tumor viruses involves crossing over.

If recombinants arose by reassortment of segments in a haploid genome, these recombinants should show only a limited number of fingerprint patterns. In recombinants selected for the same markers, the two segments containing these markers must be the same. Sequence diversity could still be caused by genome segments not carrying the selected markers, and of these there are at most two, to give a total of four segments per genome (4). The number of possible variations is then four; however, if there are only three segments per genome the same recombinants could occur in only two fingerprint variations. Since we have already observed four distinct fingerprint patterns in the PR-B x RAV-3 cross, our data would agree with reassortment only if the genome has four (but not three or two) genetically unique segments. Even in the case of four segments there is only a 9% chance for finding all four possible fingerprint patterns in the first four recombinants tested.

Disregarding our suggestive evidence on polyploidy, the remaining data on recombinant RNAs could be reconciled with reassortment, if we make the *ad hoc* assumption that class *b* RNA of leukosis virus is augmented by cellular RNA sequences to yield a class *a* molecule, when it becomes incorporated into a sarcoma virus in the process of recombination. This augmentation by cellular RNA would have to be genetically stable. Such a process could generate the observed size and sequence diversity and cannot be definitely ruled out on the basis of present data.

Is host-modification involved in the formation of recombinant RNA?

Small differences in the size of class *a* RNA can be observed among recombinants selected for the same markers. These differences could result from host modification of the viral RNA rather than from unequal crossing over. Such modifications may include various degrees of polyadenylation (15) or addition of cellular sequences acquired at the chromosomal sites at which viral DNA is thought to integrate into cellular DNA. Although such changes could account for the electrophoretic differences observed among different recombinant RNAs, it is unlikely that these variations in size amounting to only ±2% of the RNA are also responsible for the changes observed in fingerprints patterns.

Alternatively recombination between exogenous tumor viruses could include interactions with endogenous tumor viruses present in all normal chicken cells (16). This type of "host-modification" has not been tested for by our experiments. However, recombination with endogenous virus has been observed only in helper factor positive cells, in which endogenous virus is at least partially expressed (3) but not in the helper factor negative cells used to prepare our recombinants. Also, if genetic interactions with an endogenous virus were responsible for some of the new properties of recombinant RNAs, it would be difficult to explain why such modification of RNA is not regularly observed in single infection.

What is the mechanism of tumor virus recombination?

No direct answer can be given to this question from our experiments, except that crossing over appears to occur. Since there is no precedent and no plausible molecular mechanism for high frequency crossing over between viruses containing single-stranded RNA, it appears likely that recombination among avian RNA tumor viruses involves

the synthesis of the DNA provirus (17,18). The high frequency recombination among RNA tumor viruses could then be a direct consequence of polyploidy. The progeny of a doubly infected cell would be largely heterozygous, containing different genomes in a 60-70S complex. Transcription of such a heterozygous RNA into DNA would bring homologous DNAs together and could increase the chances of crossing over (3,6,17).

List of Virus Abbreviations

PR-A:	Prague Rous sarcoma virus, subgroup A.
PR-B:	Prague Rous sarcoma virus, subgroup B.
PR-C:	Prague Rous sarcoma virus, subgroup C.
*td*PR-C:	Transformation defective derivative of PR-C.
RAV-1:	Rous associated virus, type 1, subgroup A.
RAV-2:	Rous associated virus, type 2, subgroup B.
RAV-3:	Rous associated virus, type 3, subgroup A.

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